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Ultrasonic-based protein quantitation by ^{18}O-labeling: optimization and comparison between different procedures

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Herein we report results regarding the optimization and comparison between different ultrasonic-based procedures for protein quantitation by the direct ^{18}O-labeling approach. The labeling procedure was evaluated using different proteins, different ultrasonic devices and different reaction times: from 30 s to 10 min with the ultrasonic probe and from 30 s to 30 min with the sonoreactor. Variables such as the enzyme-to-protein ratio and protein concentration were also assessed. The results show that it is possible to accelerate the labeling reaction from 12 h to only 15 min with the sonoreactor without compromising the labeling efficiency. A larger variation in the double labeling yield was obtained among the different peptides, but the values for the smaller peptides are similar to the ones achieved with the classic methodology. These findings were further confirmed by labeling a complex protein mixture from human plasma. It was also found that the labeling reaction is affected by the sample concentration, even when performed with the classic overnight procedure. Copyright © 2010 John Wiley & Sons, Ltd.

The proteome of a living organism is the result of gene expression but unlike the genome, it is highly dynamic and influenced by cellular conditions and physiological states. In order to study the protein components of biological systems we need to obtain not only information about the presence or absence of proteins (qualitative information), but we also need to infer about protein expression level (quantitative information).[1]

Mass spectrometry (MS) is nowadays an essential technique in the proteomics field and when coupled with stable isotopic labeling (SIL) methods, MS can provide important quantitative information.[2–5] There are several SIL methodologies, e.g.: stable isotope labeling by amino acids in cell culture (SILAC),[6] isotope-coded affinity tags (ICAT),[7] and isobaric tag for relative and absolute quantitation (iTRAQ),[8] but the ^{18}O-enzymatic labeling of proteins is one of the most commonly used methods because it is a relatively cheap technique, easy to perform and versatile.[9–13]

In the normal ^{18}O-labeling workflow one sample is labeled in ^{18}O-enriched water while the other is labeled in natural abundance ^{16}O water. Then, the two samples are mixed and analyzed by MS. Finally, the relative abundance of each sample is calculated based on the relative intensities of the 'light' and 'heavy' labeled peptides provided by the mass spectrum.[14] The labeling reaction occurs during the hydrolysis of the peptide bond and, depending on the enzyme and on the reaction conditions, one or two ^{18}O-atoms from the $H_2{}^{18}O$-enriched medium are incorporated at the C-terminal carboxyl group of the peptide.[9] Trypsin can catalyze the incorporation of two ^{18}O-atoms, resulting in the ideal mass shift of +4 Da for the labeled peptide fragment, which is the minimum mass gap required to avoid naturally occurring isotopic interferences (e.g. ^{13}C, ^{15}N, ^{34}S) or isotopic overlapping between labeled and unlabeled species in the mass spectrum. There are two main approaches for the ^{18}O-isotopic labeling of proteins: (i) the isotopic labeling occurs during the enzymatic digestion in $H_2{}^{18}O$ buffer medium, the direct labeling procedure; or (ii) the post-digestion approach, the decoupling procedure, where the proteins are first digested in $H_2{}^{16}O$ buffer medium, dried and then labeled in $H_2{}^{18}O$ in the presence of trypsin.[15] The post-digestion approach has the advantage of consuming less $H_2{}^{18}O$, an expensive reagent, and it provides better ^{18}O conversion at the C-terminus of the peptide, thus increasing the labeling efficiency. However, the procedures used with this approach are generally longer, more elaborate and labor-intensive than the direct labeling protocols.[16] Due to these disadvantages, and because the direct labeling is done in one single step, minimizing technical variations and facilitating on-line approaches for MS protein quantitation, we chose this strategy for protein labeling in this study.

Despite the advantages of ^{18}O-labeling over other labeling techniques, there are also some drawbacks that can affect the labeling efficiency, such as: (i) variable ^{18}O-incorporation, i.e. single or double ^{18}O-incorporation at the peptide's C-terminus; and (ii) back-exchange reaction, i.e. the post-labeling exchange of ^{18}O from the peptide's C-terminal carboxyl group with ^{16}O from medium contamination with residual $H_2{}^{16}O$. In order to improve the double ^{18}O-incorporation and diminish the effect of the back-exchange reaction in the labeling efficiency, several reaction parameters such as (i) the pH of the labeling reaction, (ii) the $H_2{}^{16}O/H_2{}^{18}O$ ratio, and (iii) the residual enzymatic activity need to be optimized and controlled.[12,13,17–19] The time-

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consuming labeling reaction, 12 to 48 h, also creates a hurdle for the application of this methodology to a wider range of protein quantitation experiments.

Ultrasonic energy has been used in proteomic methodologies to enhance protein enzymatic digestion from overnight to minutes.[20,21] More recently, we reported the application of ultrasonic energy to accelerate the ^{18}O-labeling procedure with promising results. Several proteins were labeled in only 30 min in an ultrasonic bath (USB) and similar $^{16}O/^{18}O$ ratios to the classical approach (12 h labeling) were obtained. However, acceptable $^{18}O_1/^{18}O_2$ ratios were only obtained for bovine serum albumin (BSA).[22]

In this work, the application of direct ultrasonication with the ultrasonic probe and indirect ultrasonication with the sonoreactor (UTR) to enhance the enzymatic ^{18}O-labeling reaction is compared and reported. The influence of the type of ultrasonic device, the ultrasonication time, the protein concentration and the enzyme concentration in the labeling efficiency and in the ^{18}O-incorporation degree were assessed, and compared with the results previously obtained with the classical approach (overnight labeling) and with the ultrasonic bath.[22]

EXPERIMENTAL

Apparatus

Protein digestion/labeling was performed in 0.5 mL safe-lock tubes (Eppendorf, Hamburg, Germany). A minicentrifuge-vortex model Sky Line (ELMI, Riga, Latvia) and a model Spectrafuge-mini minicentrifuge (Labnet, Madrid, Spain) were used during the sample treatment. Milli-Q natural abundance ($H_2^{16}O$) water was obtained from a Simplicity™ 185 model (Millipore, Milan, Italy). A UTR200 sonoreactor (200 W, 24 kHz) and a UP100H ultrasonic probe (100 W, 30 kHz, 0.5 mm diameter probe tip; Hielscher Ultrasonics, Teltow, Germany) were used to accelerate enzymatic protein digestion/labeling.

Standards and reagents

Bovine serum albumin (BSA; 66 kDa, >97%), α-lactalbumin (14.4 kDa, ≥85%), ovalbumin (45 kDa), human plasma (lyophilized powder) and trypsin (proteomics grade) used throughout the experiment were from Sigma (Steinheim, Germany) as well as the DL-dithiothreitol (DTT, 99%) and iodoacetamide (IAA) used for protein reduction and alkylation, respectively. Ammonium bicarbonate buffer (AmBic, pH 8.5, ≥99.5%) and formic acid (FA, ~98%) were from Fluka (Buchs, Switzerland), and the $H_2^{18}O$ (95 atom %) used for protein isotopic labeling was from ISOTEC™ (Miamisburg, OH, USA). α-Cyano-4-hydroxycinnamic acid (α-CHCA, ≥99.0%), acetonitrile (99.9%) and trifluoroacetic acid (TFA, 99%) were from Fluka, Sigma-Aldrich and Riedel-de Haën (Seelze, Germany), respectively. The ProteoMass™ Peptide MALDI-MS calibration kit (MSCAL2) from Sigma was used as mass calibration standard for MALDI-TOF-MS.

Sample treatment

Protein digestion/labeling

Protein digestion/labeling was performed as previously described.[22] Briefly, stock solutions of BSA, ovalbumin and α-lactalbumin (100 pmol/μL) were prepared in AmBic (100 mM) using natural abundance water, and the protein samples were reduced with DTT (10 mM) during 1 h at 37°C, and alkylated with IAA (50 mM) in the dark at room temperature during 45 min. Aliquots of 10 μL were diluted to 100 μL with AmBic (100 mM) prepared in natural abundance water, or in 95% ^{18}O-enriched water. Trypsin (2 μL) was added to the protein samples to a final concentration of 0.47 pmol/μL and the enzymatic digestion/labeling was accelerated with: (i) ultrasonic probe operating at 50% amplitude with a 0.5 mm probe, during 30, 60, 120, 300 s and 10 min; (ii) sonoreactor (UTR) operating at 50% amplitude during 30, 60, 120, 300 s, and 10, 15 and 30 min. The enzymatic reaction was stopped after the addition of 5 μL of formic acid (50%, v/v). All the experiments were done in replicates of three (n = 3).

Enzyme-to-protein ratio effect on the ^{18}O-labeling reaction

The sample treatment before protein digestion/labeling was performed as described in the previous section. Following protein reduction and alkylation, aliquots of 10 μL of BSA (60 μg) were diluted to 100 μL with AmBic (100 mM) prepared in natural abundance water, or in 95% ^{18}O-enriched water. After the addition of 2 μL of trypsin the reaction was performed during 15 min in the sonoreactor (50% amplitude). Different enzyme-to-protein (E:P) ratios were used for protein digestion/labeling: (i) 1:120 w/w (trypsin – 0.5 μg); (ii) 1:80 w/w (trypsin – 0.75 μg); (iii) 1:60 w/w (trypsin – 1.0 μg); (iv) 1:40 w/w (trypsin – 1.5 μg); 1:30 w/w (trypsin – 2.0 μg). Formic acid 50% (v/v) (5 μL) was added to stop the enzymatic digestion.

^{18}O-labeling in low concentration protein samples

Protein reduction and alkylation was performed as described above. BSA samples of 2.5; 5; 15; 30 and 60 μg were digested/labeled in 100 μL of AmBic (100 mM) prepared in natural abundance water, or in 95% ^{18}O-enriched water. The E:P ratio used throughout these experiments was always 1:40 (w/w) except for the 2.5 μg samples, which were also digested/labeled with different E:P ratios: 1:40 w/w (trypsin – 0.0625 μg); 1:20 w/w (trypsin – 0.125 μg); 1:6.7 w/w (trypsin – 0.375 μg); and 1:3.3 w/w (trypsin – 0.75 μg). The enzymatic reaction was performed in 15 min with the sonoreactor (50% amplitude), and stopped after the addition of 5 μL of formic acid (50% v/v).

^{18}O-labeling of proteins from human plasma

Lyophilized human plasma was dissolved in 1 mL of phosphate-buffered saline (PBS) buffer (pH 7.2). Aliquots of 100 μL were precipitated overnight at −20°C with 5 volumes of cold acetone. The samples were then centrifuged at 10 000 *g* during 30 min (4°C); the supernatant was discarded and the pellet resuspended in 50 μL of AmBic (100 mM). Protein reduction and alkylation was performed as described above, and then aliquots of 10 μL were diluted to 100 μL with AmBic (100 mM) prepared in natural abundance water, or in 95% ^{18}O-enriched water. Trypsin (1 μg) was added and the enzymatic digestion/labeling

reaction allowed to proceed for 15 min in the sonoreactor at 50% amplitude, or overnight at 37°C. The enzymatic reaction was stopped after the addition of 5 μL of formic acid (50%, v/v). All the experiments were done using three replicates (n = 3).

MALDI-TOF-MS analysis

Before MS analysis, samples were mixed in a 1:1 ratio with the matrix solution of α-CHCA (10 μg/μL) prepared in 50% acetonitrile/0.1% TFA. Each sample (1 μL) was hand-spotted onto a MALDI-TOF-MS stainless steel 96-well plate and allowed to dry. The mass spectra were obtained with a Voyager DE-PRO Biospectrometry Workstation (Applied Biosystems, Foster City, USA), equipped with a nitrogen laser radiating at 337 nm. Measurements were carried out in the reflector positive ion mode, with an accelerating voltage of 20 kV, 75.1% of grid voltage, 0.002% of guide wire and a delay time of 100 ns. The monoisotopic peaks of bradykinin, angiotensin II, P14R and ACTH peptide fragments (*m/z* $[M+H]^+$: 757.3997, 1046.5423, 1533.8582 and 2465.1989, respectively) were used for the external calibration of the mass spectra. A total of 250 laser shots were summed per spectrum.

Deconvolution of MALDI mass spectra

The mass spectra deconvolution was performed with the Data Explorer™ software (version 4.0) from Applied Biosystems. This software has an advanced peak filtering method, the deisotope function, that uses a deisotoping algorithm to determine the relative abundance of multiple components with overlapping isotope distributions.[23] The deisotope function deconvolutes the mass spectra and reduces the isotopic cluster to a centroided plot composed of the monoisotopic peaks from the peak list. Additionally, for comparative purposes in order to test the correct applicability of this function, the mathematical algorithm for deconvolution described by Yao and coworkers was also used in the first steps of this work.[24]

RESULTS AND DISCUSSION

The ^{18}O-labeling reaction can incorporate one or two ^{18}O-atoms at the terminal carboxylic group of the peptide, shifting the mass value of the naturally occurring isotopic distribution by +2 or +4 Da. Figure 1 presents two theoretical cases which can occur when performing protein ^{18}O-labeling quantitation. Spectrum (a) represents the theoretical result obtained when the labeling reaction is complete and all the peptides are double labeled. Here, when mixing the labeled sample with the unlabeled control sample, there is no isotopic overlapping in the mass spectrum between the two peptide forms. Therefore, protein relative quantitation can simply be done by measuring the relative intensities of the monoisotopic peaks of each peptide. In case (b) the labeling reaction is incomplete and a mixture of single and double labeled peptides is generated, producing an isotopic overlap between the non-labeled control sample and labeled sample. This variable ^{18}O-incorporation affects the measuring of the relative abundances of the peptide and increases the error in the calculation of the correct ^{16}O/^{18}O peptide ratios.[3,11,13] Therefore, when performing ^{18}O-labeling it is important to consider not only the labeling efficiency ($^{18}O_{total}$ %), which measures the percentage of both single and double labeled peptides, but also the labeling degree ($^{18}O_2$ %) which measures the percentage of double labeled peptides.

^{18}O-labeling with direct ultrasonication

The ultrasonic probe provides ultrasonic energy directly into the reaction media and the ultrasound intensity is at least 1500 times higher than that provided by the ultrasonic bath.[25] Previous studies reported the use of direct ultrasonication to accelerate the ^{18}O-labeling reaction in a post-digestion approach with similar results to the ones obtained with the conventional protocols.[26] However, despite accelerating the enzymatic reactions up to only a few minutes, this workflow still remains tedious and long due to the several drying steps required, which increase sample losses and preclude on-line labeling approaches. Therefore, to assess the ultrasonic probe effect on peptide ^{18}O-labeling using the direct labeling approach, aliquots of BSA (60 μg) were digested in the presence of $H_2{}^{18}O$ and

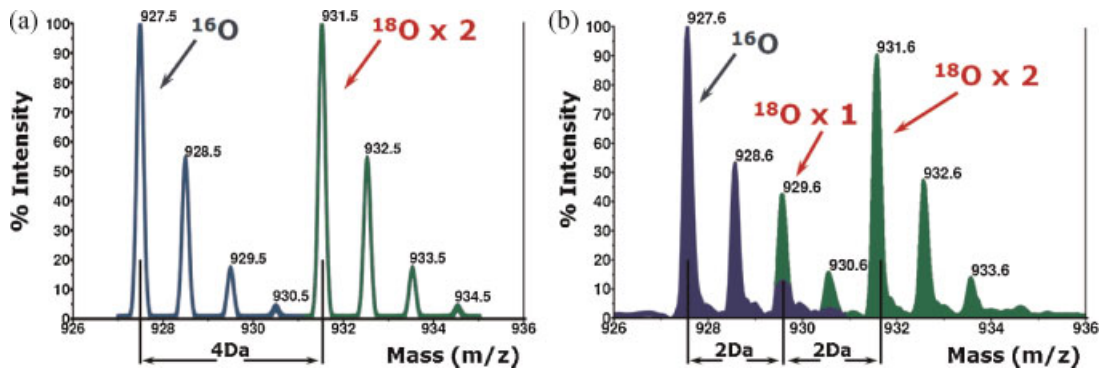

Figure 1. Complete ^{18}O-labeling vs. variable ^{18}O-labeling. (a) Complete ^{18}O-labeling – theoretical MALDI-TOF mass spectrum of a mixture (1:1) of the unlabeled (927 *m/z*) and the double labeled (931 *m/z*) peptide $(YLYEIAR)H^+$ from BSA. (b) Variable ^{18}O-labeling – theoretical MALDI-TOF mass spectrum of a mixture (1:1) of the unlabeled (927 *m/z*) and the single (929 *m/z*) and double labeled (931 *m/z*) peptide $(YLYEIAR)H^+$ from BSA.

trypsin (1 μg) with a 0.5 mm sonotrode. The ultrasonication time was between 30 s and 10 min.

Table 1 presents the labeling degree results ($^{18}O_2$ %) obtained for several BSA peptides after ultrasonication with the ultrasonic probe. In general, the labeling degree increased with the ultrasonication time reaching a maximum value for t = 120 s, and no improvements were achieved when ultrasonication was performed during 5 and 10 min. In fact, the ultrasonication of liquid media with the ultrasonic probe during long periods of time (>2 min) leads to sample overheating and aerosol formation, diminishing the efficiency of the ultrasonic treatment.[27] In addition, in a previous study developed by our group, we demonstrated that 30 s of ultrasonication with the ultrasonic probe does not affect enzyme activity, but after 60 s the activity decreases ca. 20% (casein hydrolysis with protease XIV). An ultrasonication time of 120 s led to the complete inactivation of the enzyme.[28] The results obtained also show that the double ^{18}O-labeling yield decreased with the increasing mass of the peptide fragment. The smallest peptide fragment considered in this study (YLYEIAR)H^+ – 927 *m/z* was double labeled with an efficiency of 63.9% (t = 120 s) whilst the larger peptide (KVPQVSTPTLVEVSR)H^+ – 1639 *m/z* presented a double labeling efficiency of only 12.6% for the same ultrasonication time. Considering that the oxygen exchange rate is dependent on the peptide size, sequence and type of amino acid,[29] it is possible that the ultrasonic energy provided by the ultrasonic probe was not sufficient to enhance the different reaction rates during the short reaction times tested here, since when the labeling reaction was performed during 12 h at 37°C the double labeling yield was ca. 76% for all the peptides.[22]

Concerning the isotopic labeling efficiency (Table 2), i.e. the percentage of peptides labeled with at least one ^{18}O ($^{18}O_{total}$ %), the results show that the best performance was obtained for t = 120 and t = 300 s, and no significant improvement was achieved when ultrasonication was performed during 10 min. The percentage of single or double labeled peptides was higher than 90% for all peptides, except fragment (LGEYGFQNALIVR)H^+ – 1479 *m/z* with a labeling efficiency below 85% for all the ultrasonication times tested. These results are closer to the values obtained with the USB, with a labeling efficiency higher than 92% for all peptides, but they are still below the values obtained by the classic methodology (overnight labeling), with labeling efficiencies higher than 96%.[22] This is probably due to the fact that the labeling reaction is a two-step reaction. In the first step the enzyme forms an ester intermediate with the peptide and during the hydrolysis of the amide bond the first ^{18}O from the medium is incorporated at the terminal carboxyl group. During the second step of the reaction, also known as the carboxyl exchange reaction, the enzyme forms another ester intermediate with the peptide terminal carboxyl group and after a series of esterification and hydrolysis cycles the peptide will be double labeled.[29] Unfortunately, the rates of the carboxyl oxygen exchange reaction are much lower than the peptide bond hydrolysis, which lead to the variable ^{18}O-incorporation when short reaction times are used. It is also important to stress that the carboxyl exchange reaction is a reversible reaction; therefore the ultrasonic energy not only accelerates the ^{16}O exchange from the peptide's carboxyl group with ^{18}O from the medium, but also enhances the reverse reaction. The results presented in Tables 1 and 2 also suggest that a complex relation involving ultrasonic energy and the first and second ^{18}O-incorporations take place. It seems that this reaction can be enhanced with ultrasonic energy of low intensity, such as the one provided by the ultrasonic bath and the sonoreactor. Yet, a system like the ultrasonic probe, 30 times more intense than the sonoreactor and 1500 times more intense than the ultrasonic bath, seems to produce uncontrolled effects which compromise an effective ^{18}O-incorporation, especially the double ^{18}O-incorporation. The nature of these effects remains unclear.

^{18}O-labeling with the UTR

The sonoreactor (UTR) can be defined as a high-intensity ultrasonic bath.[25] Even though the ultrasonic energy generated by the sonoreactor is 30 times less intense than the one provided by the ultrasonic probe, several advantages make this device suitable for proteomic workflows, such as: (i) sample ultrasonication in sealed vials, which prevents cross-contamination between samples; (ii) no aerosol formation, which improves biosafety when working with hazardous samples from pathogenic bacteria and viruses; (iii) lower sample volume is needed for ultrasonication; (iv) high throughput, since many samples can be treated at once unlike common ultrasonic probes.[21,25]

The efficiency of the sonoreactor was first evaluated using BSA as the model protein. The results in Table 1 show that the percentage of double labeled peptides ($^{18}O_2$) increased with the ultrasonication time. For the time range between 30 and 120 s the percentage of double labeled peptides obtained with the sonoreactor was similar to the one achieved with the ultrasonic probe. However, better results were obtained with the sonoreactor when BSA was labeled during 5 min and 10 min, especially for the largest peptides. This is in agreement with the data presented before for the ultrasonic probe, where it was said that the lack of improvement in the labeling degree efficiency for larger ultrasonication times could be related to aerosol formation, sample overheating and unexpected reactions caused by the high ultrasound intensity provided by the probe. When ultrasonication is performed with the sonoreactor the aerosol formation is insignificant and the temperature of the water bath can be controlled. In addition the ultrasound intensity provided is 30 times lower than the one obtained with the ultrasonic probe. Therefore, no sample overheating or spreading through the walls of the container occurs, and the ultrasonic efficiency is maintained during the process. Regarding the larger ultrasonication times tested, 15 and 30 min, the ^{18}O-labeling degree obtained with the sonoreactor was higher than when the ultrasonic bath was used, and for the smallest peptide fragments, (YLYEIAR)H^+ – 927 *m/z*; (ALKAWSVAR)H^+ – 1001 *m/z*; and (RHPEYAVSVLLR)H^+ – 1439 *m/z*, the percentages of double ^{18}O-incorporation were higher and close to the values obtained with the classic overnight protocol (76%).

The efficiency of the ^{18}O-labeling reaction (single and double labeling) also increased with the ultrasonication time (Table 2). Like before, the results obtained with the sonoreactor between 30 s and 5 min were similar to the ones obtained with the ultrasonic probe, but with 10 min of ultrasonication the labeling efficiency was higher for the sonoreactor. As previously referred, this result suggests that the labeling reaction is more effective when ultrasonic energy

Table 1. Percentage of double ^{18}O-incorporation ($^{18}O_2$) at the peptide's C-terminus. BSA (60 μg) digestion/labeling with trypsin was performed during 12 h (overnight) and with different ultrasonic devices: (i) ultrasonic bath (USB 130 kHz) operating at 70% amplitude and 60°C; (ii) ultrasonic probe (UP) operating at 50% amplitude and equipped with a 0.5 mm sonotrode; (iii) sonoreactor (UTR) operating at 50% amplitude. The sonication time was comprised between 30 s and 30 min. The five most intense mass peaks were considered: 927.49 *m/z* – (YLYEIAR)H^+; 1001.59 *m/z* – (ALKAWSVAR)H^+; 1439.81 *m/z* – (RHPEYAVSVLLR)H^+; 1479.80 *m/z* – (LGEYGFQNALIVR)H^+; 1639.94 *m/z* – (KVPQVSTPTLVEVSR)H^+ (n = 3)

$^{18}O_2$ %

$[M+H]^+$ (*m/z*)	Sonication time								
	30 s	60 s	120 s	300 s	10 min	15 min	30 min	12 h	Method
927.49	—	—	—	—	—	—	—	75.61 ± 2.30	**Overnight***
	—	—	—	—	—	70.88 ± 2.56	70.57 ± 0.53	—	**USB 130 kHz***
	42.88 ± 4.32	48.97 ± 12.08	63.91 ± 6.99	61.29 ± 2.76	51.81 ± 5.37	—	—	—	**UP**
	45.98 ± 7.69	47.72 ± 2.23	58.50 ± 4.90	63.66 ± 3.25	73.17 ± 1.04	74.75 ± 0.01	74.10 ± 0.36	—	**UTR**
1001.59	—	—	—	—	—	—	—	**	**Overnight***
	—	—	—	—	—	71.67 ± 1.57	75.67 ± 5.34	—	**USB 130 kHz***
	29.47 ± 1.86	40.78 ± 13.83	59.09 ± 9.80	58.97 ± 3.30	56.17 ± 7.77	—	—	—	**UP**
	33.99 ± 5.48	37.82 ± 2.96	54.48 ± 5.78	62.73 ± 3.77	75.12 ± 5.82	71.72 ± 10.03	**	—	**UTR**
1439.81	—	—	—	—	—	—	—	77.75 ± 3.28	**Overnight***
	—	—	—	—	—	56.60 ± 2.18	56.73 ± 0.20	—	**USB 130 kHz***
	9.03 ± 3.22	16.12 ± 12.57	34.35 ± 13.04	29.94 ± 7.23	29.48 ± 1.64	—	—	—	**UP**
	14.97 ± 7.31	15.13 ± 1.95	30.36 ± 1.50	35.42 ± 6.51	60.89 ± 0.30	69.86 ± 1.97	72.29 ± 1.98	—	**UTR**
1479.80	—	—	—	—	—	—	—	74.70 ± 2.02	**Overnight***
	—	—	—	—	—	46.63 ± 0.88	45.62 ± 5.38	—	**USB 130 kHz***
	24.31 ± 3.55	13.17 ± 2.06	30.52 ± 6.73	23.11 ± 4.84	17.45 ± 3.09	—	—	—	**UP**
	34.72 ± 3.59	22.26 ± 1.76	27.85 ± 3.12	31.41 ± 1.96	52.18 ± 0.29	58.63 ± 5.79	66.92 ± 1.17	—	**UTR**
1639.94	—	—	—	—	—	—	—	76.73 ± 0.01	**Overnight***
	—	—	—	—	—	21.72 ± 0.86	24.67 ± 0.78	—	**USB 130 kHz***
	**	**	12.59 ± 4.11	15.52 ± 12.09	**	—	—	—	**UP**
	**	**	9.44 ± 2.23	10.38 ± 8.15	30.30 ± 4.67	41.12 ± 2.48	42.61 ± 2.07	—	**UTR**

*Results previously obtained for the overnight and USB 130 kHz digestion/labeling were used for comparative purposes.[22]
** Peptide not present in the spectra.

Rapid Communications in Mass Spectrometry

Table 2. Percentage of total ^{18}O-incorporation ($^{18}O_{total}$) at the peptide's C-terminus. BSA (60 μg) digestion/labeling with trypsin was performed during 12 h (overnight) and with different ultrasonic devices: (i) ultrasonic bath (USB 130 kHz) operating at 70% amplitude and 60°C; (ii) ultrasonic probe (UP) operating at 50% amplitude and equipped with a 0.5 mm sonotrode; iii) sonoreactor (UTR) operating at 50% amplitude. The sonication time was comprised between 30 s and 30 min. The five most intense mass peaks were considered: 927.49 *m/z* – (YLYEIAR)H^+; 1001.59 *m/z* – (ALKAWSVAR)H^+; 1439.81 *m/z* – (RHPEYAVSVLLR)H^+; 1479.80 *m/z* – (LGEYGFQNALIVR)H^+; 1639.94 *m/z* – (KVPQVSTPTLVEVSR)H^+ (n = 3)

	$^{18}O_{total}$ %								
	Sonication time								
$[M+H]^+$ (*m/z*)	30 s	60 s	120 s	300 s	10 min	15 min	30 min	12 h	Method
927.49	—	—	—	—	—	—	—	**98.55 ± 1.80**	**Overnight***
	—	—	—	—	—	97.53 ± 0.54	97.60 ± 1.29	—	**USB 130 kHz***
	92.85 ± 0.33	93.59 ± 1.90	96.07 ± 1.10	96.44 ± 0.19	94.19 ± 1.76	—	—	—	**UP**
	93.54 ± 0.47	92.27 ± 0.23	95.57 ± 1.02	96.56 ± 0.22	97.54 ± 0.01	98.66 ± 0.01	99.23 ± 1.09	—	**UTR**
1001.59	—	—	—	—	—	—	—	**	**Overnight***
	—	—	—	—	—	96.93 ± 3.36	94.40 ± 4.33	—	**USB 130 kHz***
	89.76 ± 0.81	92.25 ± 3.06	95.95 ± 2.32	95.36 ± 0.95	91.52 ± 2.66	—	—	—	**UP**
	89.28 ± 0.67	89.19 ± 0.51	93.85 ± 0.59	96.46 ± 0.08	95.75 ± 6.01	97.12 ± 4.08	***	—	**UTR**
1439.81	—	—	—	—	—	—	—	96.35 ± 0.50	**Overnight***
	—	—	—	—	—	95.50 ± 1.86	94.64 ± 1.02	—	**USB 130 kHz***
	88.44 ± 1.14	89.75 ± 1.94	91.36 ± 2.12	90.10 ± 0.87	89.31 ± 0.49	—	—	—	**UP**
	86.97 ± 1.60	88.21 ± 0.35	91.67 ± 1.10	91.90 ± 1.42	95.73 ± 0.13	97.45 ± 0.64	96.42 ± 2.67	—	**UTR**
1479.80	—	—	—	—	—	—	—	96.60 ± 0.84	**Overnight***
	—	—	—	—	—	93.02 ± 0.97	90.21 ± 2.88	—	**USB 130 kHz***
	61.05 ± 2.50	66.21 ± 8.17	80.90 ± 8.77	82.38 ± 7.10	85.04 ± 2.92	—	—	—	**UP**
	68.52 ± 7.87	77.39 ± 2.66	82.77 ± 0.03	88.33 ± 0.78	93.22 ± 2.85	93.17 ± 1.29	97.34 ± 0.13	—	**UTR**
1639.94	—	—	—	—	—	—	—	99.80 ± 0.29	**Overnight***
	—	—	—	—	—	92.61 ± 0.21	92.52 ± 2.11	—	**USB 130 kHz***
	**	**	90.64 ± 1.51	93.08 ± 1.67	**	—	—	—	**UP**
	**	**	89.49 ± 5.59	89.81 ± 0.33	95.76 ± 5.99	93.25 ± 0.63	96.23 ± 5.34	—	**UTR**

* Results previously obtained for the overnight and USB 130 kHz digestion/labeling were used for comparative purposes.[22]
** Peptide not present in the spectra.

Table 3. Percentage of double ^{18}O-incorporation ($^{18}O_2$) and total ^{18}O-incorporation ($^{18}O_{total}$) in different peptides from ovalmunin and α-lactalbumim. The labeling reaction was performed during 12 h (overnight) and with different ultrasonic devices: (i) 30 min with the ultrasonic bath (USB 130 kHz) operating at 70% amplitude and 60°C; and (ii) 15 and 30 min with the sonoreactor (UTR) operating at 50% amplitude. The most intense peptides considered for ovalbumin were: (VYLPR)H^+ – 647.39 *m/z*; (HIATNAVLFFGR)H^+ – 1345.74 *m/z*; (GGLEPINFQTAADQAR)H^+ – 1687.84 *m/z*; and for α-lactalbumim: (CEVFR)H^+ – 710.33 *m/z*; (VGINYWLAHK)H^+ – 1200.65 *m/z* (n = 3)

Protein	$[M+H]^+$ (*m/z*)	$^{18}O_2$ %			$^{18}O_{total}$ %			Sonication time (min)
		Overnight*	USB 130 kHz*	UTR	Overnight*	USB 130 kHz*	UTR	
Ovalbumin	647.39	73.45 ± 3.49	—	65.42 ± 10.55	97.65 ± 1.58	—	98.68 ± 1.18	15
			32.19 ± 3.07	69.78 ± 1.46		91.67 ± 0.06	99.24 ± 0.11	30
	1345.74	68.86 ± 3.45	—	57.37 ± 5.71	97.15 ± 1.16	—	95.24 ± 1.71	15
			20.50 ± 1.53	66.16 ± 2.74		88.29 ± 0.90	96.59 ± 0.18	30
	1687.84	65.96 ± 2.53	—	71.60 ± 2.33	93.44 ± 5.21	—	96.41 ± 0.82	15
			24.06 ± 2.76	71.08 ± 0.18		89.92 ± 1.16	98.07 ± 0.47	30
α-Lactalbumim	710.33	76.12 ± 1.48	—	27.77 ± 0.11	97.05 ± 0.44	—	90.90 ± 0.08	15
			4.99 ± 0.89	30.17 ± 1.16		87.67 ± 0.51	91.95 ± 0.60	30
	1200.65	71.34 ± 1.02	—	15.87 ± 1.25	96.30	—	90.43 ± 0.43	15
			3.01 ± 0.12	24.02 ± 0.14		87.79 ± 2.27	90.40 ± 0.98	30

*Results previously obtained for the overnight and USB 130 kHz digestion/labeling were used for comparative purposes.[22]

of low intensity is used. This conclusion is confirmed by the data obtained with the sonoreactor and the ultrasonic bath. These devices, which provide low intensity ultrasonic energy, can accelerate the labeling reaction to the same levels as the overnight process (12 h) in just 15 min.

Additional proteins were ^{18}O-labeled to assess the efficiency of the UTR technology. Therefore, aliquots of ovalbumin and α-lactalbumin were labeled in the presence of $H_2{}^{18}O$ and trypsin during 15 and 30 min. The ultrasonication times were chosen based on the best labeling degree and efficiency obtained for BSA. As may be seen in Table 3, for the most intense peptides of ovalbumin, the labeling efficiency ($^{18}O_{total}$) was between 96 and 99% for both 15 and 30 min of ultrasonication. In addition, the labeling efficiency obtained in only 15 min with the sonoreactor was higher than that obtained during 30 min with the ultrasonic bath, and it was equal or higher than that obtained with the classic overnight reaction, which was between 93 and 97%. In terms of the yield of double labeled peptides ($^{18}O_2$), the results were mostly the same, regardless of the ultrasonication time applied: for peptide (VYLPR)H^+ – 647.39 *m/z*, the double labeling yield was between 65 and 70% with 15 and 30 min, respectively; and for the largest peptide (GGLEPINFQTAADQAR)H^+ – 1687.84 *m/z* the double labeling yield was 71% with both reaction times, which is higher than the yield obtained with the classic method, 66%.

Concerning the labeling efficiency ($^{18}O_{total}$) obtained for α-lactalbumin, the results show that at least 90% of the peptides were labeled with one or two ^{18}O-atoms for both 15 and 30 min of ultrasonication, whilst with the ultrasonic bath a labeling efficiency of 87% was obtained after 30 min. However, in this case the results were below the classic overnight procedure with a labeling efficiency of ca. 97%. As far as the double ^{18}O-incorporation is concerned, the sonoreactor performed much better than the ultrasonic bath by promoting the double ^{18}O-incorporation in 30% of α-lactalbumin's (CEVFR)H^+ (710.33 *m/z*) peptide, whereas the results obtained with the ultrasonic bath for the same peptide were 6 times lower: 5% of double ^{18}O-incorporation during 30 min of ultrasonication. However, the sonoreactor results were still lower than the ones obtained with the classic overnight methodology for which 71 to 76% of the peptides were double ^{18}O-labeled.

Influence of E:P ratio on the ^{18}O-labeling reaction

The double ^{18}O-incorporation at the terminal carboxylic group of the peptide is essential to obtain mass spectra free from isotopic overlap between unlabeled and labeled species, thus improving the precision and accuracy of the protein quantitation method. In order to achieve a complete double ^{18}O-labeling it is important to accelerate the carboxyl oxygen exchange reaction. This can be done in several different ways, such as: (i) decreasing the pH of the enzyme-catalyzed carboxyl oxygen exchange reaction from 8.5, the optimum pH for trypsin proteolytic activity, to pH between 5 and 6, where trypsin presents the best catalytic activity regarding the carboxyl oxygen exchange reaction;[15,30] (ii) or by performing the carboxyl oxygen exchange in aqueous solutions with organic solvents.[31] Despite providing promising results, these techniques rely on the post-digestion labeling approach, where the sample is first digested in

natural abundance water media, then dried and finally labeled in an appropriate buffer enriched with ^{18}O-water. Due to the extra drying steps introduced, this method requires more time, is not suitable for on-line approaches and the results might suffer from a higher technical variation. Another way to increase the yield of the double labeling is to use a higher E:P ratio, although this might be a problem due to enzyme autolysis, because of the ion suppression effect caused by enzyme peptides over the protein peptides in the mass spectra. The use of calcium salts to improve trypsin's activity and prevent autolysis has been reported,[32,33] but this implicates an extra step to remove the salts before MS analysis to avoid interferences with peptide ionization. Immobilized trypsin can also be used as an alternative to overcome the problem of back-exchange and enzyme autolysis,[16,34] but the cost of this reagent may be discouraging to some laboratories.

To further evaluate the combined effect of ultrasound and different enzyme-to-protein (E:P) ratios, aliquots of BSA (60 μg) were labeled during 15 min with the sonoreactor in the presence of $H_2{}^{18}O$ and different amounts of trypsin. The E:P ratio varied between 1:120 and 1:30 (w/w). As may be seen in Table 4, the lowest labeling efficiency ($^{18}O_{total}$ %) was obtained when only 0.5 μg (1:120 w/w E:P ratio) of trypsin was used. In this case the labeling efficiency varied from 80%, for the two larger peptides (1479 and 1639 *m/z*), to 93% for the smallest peptide (927 *m/z*). When other E:P ratios were used the labeling efficiencies obtained with each ratio were similar among them: between 96 and 98% for the smaller peptides – 927, 1001 and 1439 *m/z*; and between 93 and 96% for the largest peptides, 1479 and 1639 *m/z*. Regarding the double ^{18}O-incorporation ($^{18}O_2$) yield, the worst performance was obtained with the lowest amount of trypsin (0.5 μg): 50% of the $(YLYEIAR)H^+$ – 927 *m/z* peptides were double labeled with 15 min of ultrasonication, while the classical overnight labeling methodology achieved a double labeling yield of 75% for the same peptide. Focusing on peptide fragments $(YLYEIAR)H^+$ – 927 *m/z* and (LGEYGFQNALIVR)H+ – 1479 *m/z*, results show that the double labeling degree increased with the amount of enzyme, reaching a maximum when 1:40 (w/w) E:P ratio was used: 75 and 65%, respectively. For these peptides no improvement in the double incorporation yield was obtained when the E:P ratio was raised to 1:30 (w/w). Regarding peptide fragments $(RHPEYAVSVLLR)H^+$ – 1439 *m/z* and $(KVPQVSTPTLVEVSR)H^+$ –1639 *m/z*, the labeling degree obtained also increased with the amount of trypsin, but no significant differences were found when the E:P ratio varied from 1:60 to 1:30 (w/w). In addition, no significant interference from trypsin autolysis peptides was found in the mass spectra of the samples labeled with a higher quantity of trypsin (Fig. 2). Overall, the best results were obtained when the E:P ratio was 1:40 (w/w), which is in the range of the recommended E:P ratios by Sigma-Aldrich® for the in-solution protein digestion: between 1:100 and 1:20 (w/w).[35]

Influence of sample concentration on the ^{18}O-labeling of proteins

When the sample concentration decreases the probability of having protein and enzyme molecules to collide with each other and establish bonds also decreases, which may compromise protein digestion/^{18}O-labeling. Thus, the ultrasound effect on the isotopic labeling reaction of low concentration protein samples was evaluated: BSA samples ranging from 2.5 to 60 μg were ^{18}O-labeled during 15 min with the sonoreactor in the presence of trypsin, using an E:P ratio of 1:40 (w/w) which was previously found to be the best

Table 4. Effect of the enzyme-to-protein ratio (E:P) on the labeling efficiency ($^{18}O_{total}$ %) and labeling degree ($^{18}O_2$ %). Aliquots of BSA (60 μg) were labeled during 15 min with the sonoreactor (50% amplitude) in the presence of $H_2{}^{18}O$ and trypsin. Different E:P ratios were used: (i) 1:120 w/w (trypsin – 0.5 μg); (ii) 1:80 w/w (trypsin – 0.75 μg); (iii) 1:60 w/w (trypsin – 1.0 μg); (iv) 1:40 w/w (trypsin – 1.5 μg); (v) 1:30 w/w (trypsin – 2.0 μg). The five most intense mass peaks were considered: 927.49 *m/z* – $(YLYEIAR)H^+$; 1001.59 *m/z* – $(ALKAWSVAR)H^+$; 1439.81 *m/z* – $(RHPEYAVSVLLR)H^+$; 1479.80 *m/z* – $(LGEYGFQNALIVR)H^+$; 1639.94 *m/z* – $(KVPQVSTPTLVEVSR)H^+$ (n = 3)

	E:P ratio [Trypsin (μg)]				
	$^{18}O_2$ %				
$[M+H]^+$ (*m/z*)	1:120 [0.5]	1:80 [0.75]	1:60 [1.0]	1:40 [1.5]	1:30 [2.0]
927.49	50.46 ± 1.86	70.63 ± 2.08	74.75 ± 0.01	75.43 ± 0.51	72.63 ± 1.28
1001.59	58.98 ± 6.70	76.03 ± 2.13	71.72 ± 10.03	*	*
1439.81	24.19 ± 3.52	52.22 ± 0.22	69.86 ± 1.97	69.05 ± 1.96	68.90 ± 1.26
1479.80	23.15 ± 1.69	45.44 ± 3.40	58.63 ± 5.79	64.51 ± 0.18	58.61 ± 1.62
1639.94	6.74 ± 2.22	19.02 ± 1.45	41.12 ± 2.48	40.38 ± 7.54	42.71 ± 3.02
			$^{18}O_{Total}$ %		
927.49	93.56 ± 0.08	97.59 ± 2.49	98.66 ± 0.01	95.90 ± 2.52	97.39 ± 0.17
1001.59	93.54 ± 4.30	100	97.12 ± 4.08	*	*
1439.81	90.64 ± 1.20	95.93 ± 1.43	97.45 ± 0.64	97.61 ± 1.32	95.63 ± 1.02
1479.80	78.35 ± 7.88	94.31 ± 0.51	93.17 ± 1.29	96.78 ± 0.85	92.38 ± 0.83
1639.94	83.79 ± 3.40	92.51 ± 0.38	93.25 ± 0.63	95.53 ± 1.22	92.23 ± 2.32

*Peptide not present in the spectra.

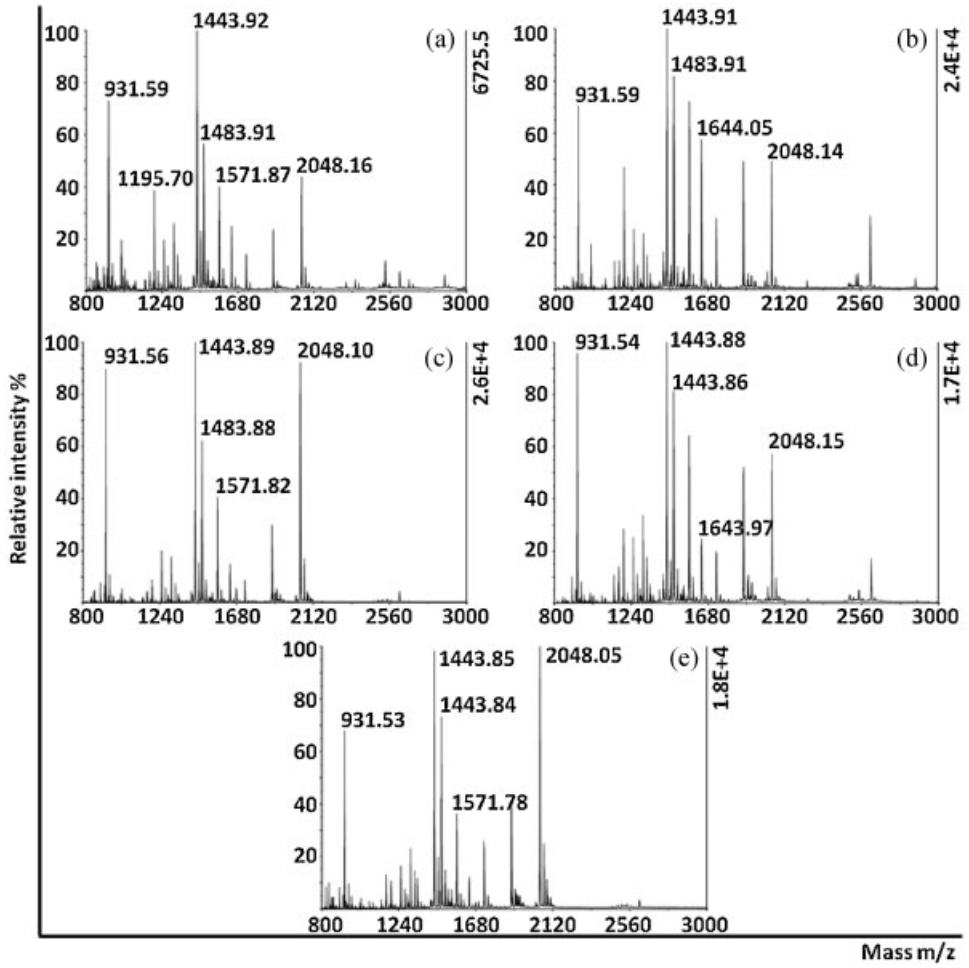

Figure 2. Effect of the enzyme-to-protein (E:P) ratio on the MALDI-TOF mass spectra obtained after protein ^{18}O-labeling with different amounts of trypsin: (a) 1:120 w/w (trypsin – 0.5 μg); (b) 1:80 w/w (trypsin – 0.75 μg); (c) 1:60 w/w (trypsin – 1.0 μg); (d) 1:40 w/w (trypsin – 1.5 μg); and (e) 1:30 w/w (trypsin – 2.0 μg).

Table 5. Effect of the sample concentration in the labeling efficiency ($^{18}O_{total}$ %) and labeling degree ($^{18}O_2$ %). Aliquots of BSA: (i) 2.5 μg; (ii) 5 μg; (iii) 15 μg; (iv) 30 μg; and (v) 60 μg were labeled during 15 min with the sonoreactor (50% amplitude) in the presence of $H_2{}^{18}O$ and trypsin. A constant enzyme-to-protein ratio was used in this experiment: 1:40 w/w. The five most intense mass peaks were considered: 927.49 *m/z* – (YLYEIAR)H^+; 1001.59 *m/z* – (ALKAWSVAR)H^+; 1439.81 *m/z* – (RHPEYAVSVLLR)H^+; 1479.80 *m/z* – (LGEYGFQNALIVR)H^+; 1639.94 *m/z* – (KVPQVSTPTLVEVSR)H^+ (n = 3)

	BSA (μg)				
	$^{18}O_2$ %				
$[M+H]^+$ (*m/z*)	2.5	5	15	30	60
927.49	25.23 ± 3.57	32.55 ± 0.88	61.48 ± 2.79	72.69 ± 1.67	75.43 ± 0.51
1001.59	25.60 ± 2.15	27.06 ± 3.69	67.98 ± 0.56	86.48 ± 6.60	*
1439.81	13.00 ± 0.44	8.26 ± 2.54	38.89 ± 0.02	54.19 ± 4.51	69.05 ± 1.96
1479.80	15.35 ± 12.92	8.80 ± 1.22	34.72 ± 2.03	48.67 ± 2.55	64.51 ± 0.18
1639.94	*	*	5.77 ± 1.16	18.17 ± 10.28	40.38 ± 7.54
			$^{18}O_{Total}$ %		
927.49	94.15 ± 0.11	92.39 ± 0.03	96.77 ± 0.55	98.35 ± 0.35	95.90 ± 2.52
1001.59	89.29 ± 3.45	91.48 ± 0.48	97.51 ± 1.07	100.00	*
1439.81	90.05 ± 7.44	92.29 ± 1.80	92.31 ± 0.08	95.25 ± 0.65	97.61 ± 1.32
1479.80	86.04 ± 4.48	83.17 ± 1.77	87.50 ± 2.63	95.48 ± 1.10	96.78 ± 0.85
1639.94	*	*	90.74 ± 2.52	91.89 ± 0.80	95.53 ± 1.22

* Peptide not present in the spectra.

working E:P ratio. The results in Table 5 show that the labeling efficiency ($^{18}O_{total}$) and the yield of double labeled peptides ($^{18}O_2$) were higher when the protein concentration used was 0.6 μg/μL (60 μg of BSA). Regarding the smallest BSA peptide, (YLYEIAR)H^+ – 927 *m/z*, the variation in the labeling efficiency was between 94 and 96% for the lowest (0.025 μg/μL) and highest (0.6 μg/μL) protein concentration samples, respectively. Yet, the variation obtained in the percentage of double labeled peptides between the two protein samples was much higher: 25% of the peptides were double labeled in the 0.025 μg/μL BSA samples, in contrast with the 75% yield obtained when 0.6 μg/μL of BSA was used. The same pattern was observed for the other peptides: the labeling efficiency and the labeling degree increased with the increasing sample concentration.

These results suggest that the ultrasonic energy provided by the sonoreactor is suitable for the enhancement of the peptide bond hydrolysis, but not for the acceleration of the carboxyl oxygen exchange reaction in samples of low protein concentration. Thus, in order to achieve a better double labeling yield at the low concentration range using only 15 min of ultrasonication, we increased the E:P ratios from 1:40 w/w (trypsin – 0.0625 μg) to 1:3.33 w/w (trypsin – 0.75 μg) in the isotopic labeling of 2.5 μg of BSA. The overnight labeling reaction was also performed for comparative purposes. The results in Fig. 3 show that the percentage of double ^{18}O-incorporation ($^{18}O_2$) obtained with the sonoreactor (15 min) increased with the E:P ratio. Yet, a labeling yield higher than 70% was only achieved for the smallest peptides, 927 *m/z* and 1001 *m/z*, when the E:P ratio was 1:3.3 (w/w). Interestingly, the results achieved with the 12 h labeling method also presented some variation, especially for the larger peptides. Considering peptides (LGEYGFQNALIVR)H^+ – 1479 *m/z* and (KVPQVSTPTLVEVSR)H^+ –1639 *m/z*, the labeling efficiency ($^{18}O_{total}$ %) was between 92 and 98%, but the double labeling yield ($^{18}O_2$ %) was only superior to 70% for the E:P ratios higher than 1:6.7 (w/w). It is important to note that these E:P ratios are much higher than the recommended ones.[35] Therefore, when working with samples of low protein concentration, the peptides chosen for protein quantitation are of special importance, as well as the total time for labeling. In order to achieve the maximum double ^{18}O-incorporation yield the reaction time must probably be increased beyond the 12 h, if no ultrasonication is used, or smaller peptides should be chosen for protein quantitation. Protein concentration strategies, like protein precipitation or ultrafiltration methods, might also be adopted in complex protein samples in order to increase sample concentration.

Ultrasound-based ^{18}O-labeling of proteins from human plasma

The labeling procedure reported in this manuscript was further tested in a complex protein sample from human plasma. As we were only interested in studying the labeling efficiency, protein identification was not performed. Thus, after precipitation with cold acetone, protein aliquots of 10 μL in ammonium bicarbonate (100 mM, pH 7.5–8.5) were reduced, alkylated and finally labeled with trypsin in ^{18}O- or ^{16}O-enriched buffer by two different methods: (i) overnight (37°C) and (ii) in the sonoreactor during 15 min (50% amplitude), which was previously found to be the best ultrasonic enhanced ^{18}O-labeling method. Regarding the

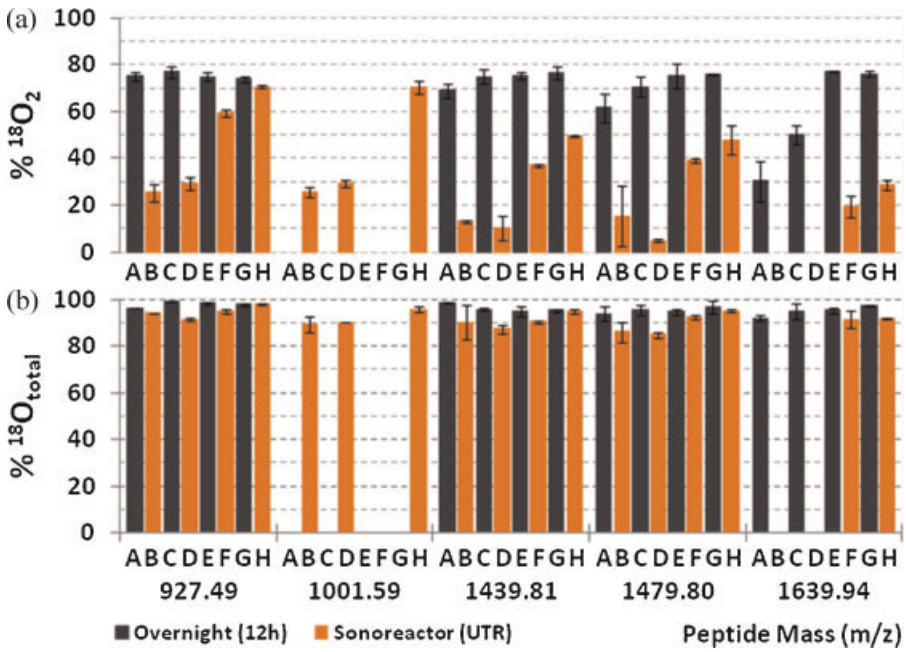

Figure 3. Effect of the sample concentration on the labeling efficiency ($^{18}O_{total}$ %) and labeling degree ($^{18}O_2$ %). Aliquots of BSA (2.5 μg) were labeled with increasing amounts of trypsin during 12 h (overnight) at 37°C, and 15 min with the sonoreactor (50% amplitude): (A) overnight labeling with 0.0625 μg trypsin; (B) sonoreactor labeling with 0.0625 μg trypsin; (C) overnight labeling with 0.125 μg trypsin; (D) sonoreactor labeling with 0.125 μg trypsin; (E) overnight labeling with 0.375 μg trypsin; (F) sonoreactor labeling with 0.375 μg trypsin; (G) overnight labeling with 0.75 μg trypsin; and (H) sonoreactor labeling with 0.75 μg trypsin (n = 3).

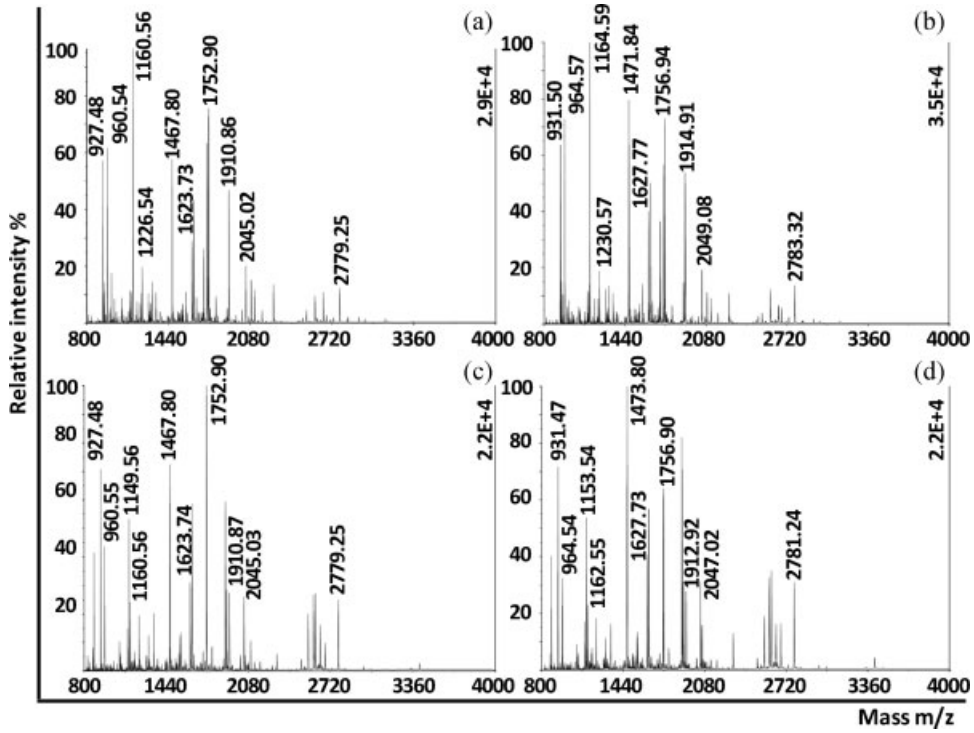

Figure 4. ^{18}O-labeling of complex protein samples from human plasma. Spectra (a) and (b) correspond to the overnight protein digestion at 37°C with trypsin in ^{16}O- and ^{18}O-enriched buffer, respectively. Spectra (c) and (d) correspond to protein digestion with the sonoreactor (15 min; 50% amplitude) in ^{16}O- and ^{18}O-enriched buffer (for details, see section *^{18}O-labeling of proteins from human plasma*).

number of peptides obtained with the different procedures, 181 peptides were labeled with the overnight method, 177 peptides were labeled with the sonoreactor and, from these peptides, 122 were common to both methods. The remaining peptides, which were characteristic to each method, had a relative intensity below 15%. Furthermore, as can be seen in Fig. 4, the background noise and baseline in the mass spectra obtained with the different labeling methods were similar.

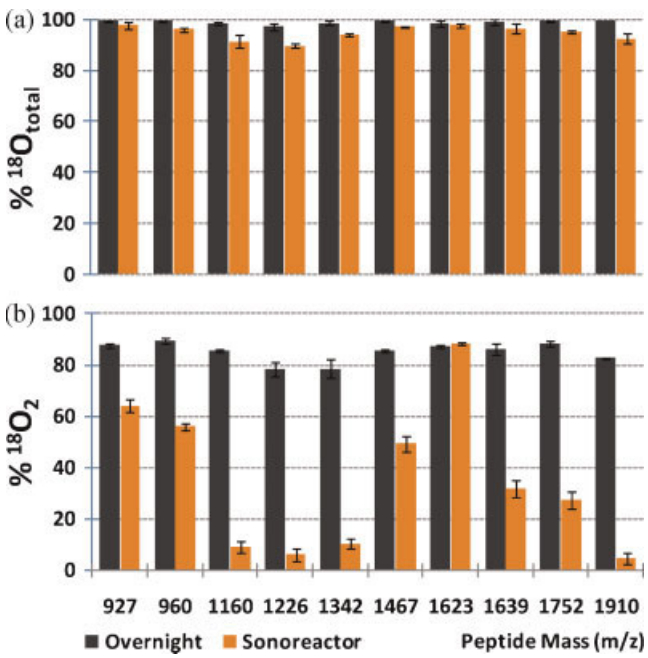

Figure 5. ^{18}O-labeling of complex protein samples from human plasma. Comparison between: (a) ^{18}O-labeling efficiency ($^{18}O_{total}$ %) and (b) ^{18}O-labeling degree ($^{18}O_2$ %) obtained with the overnight (12 h; 37°C) and the sonoreactor (15 min; 50% amplitude) methodologies (n = 3).

Regarding the labeling efficiency for the most intense peptides, the results obtained were similar between the two methodologies tested and showed that all the peptides were labeled with at least one ^{18}O-atom in a percentage higher than 90% when the sonoreactor methodology was used (Fig. 5(a)). These results are also very close to the best results obtained previously for the standard proteins. However, as far as the labeling degree is concerned (Fig. 5(b)), the results presented a larger variation between different peptides, as obtained for α-lactalbumin when the accelerated procedure was used. In fact, only the peptide corresponding to 1623 *m/z* was double labeled with a similar percentage to the overnight procedure: ca. 88%. Peptides corresponding to 927, 960 and 1467 *m/z* present double labeling percentages higher than 50%, but lower than the double labeling yield of 85% obtained with the overnight methodology. This is probably related to the presence of multiple proteins with different characteristics, some of them more efficiently digested with trypsin than others. It must be also noted that peptides corresponding to 1160, 1226 and 1342 *m/z* were present in the mass spectra of the ultrasonicated samples with a lower relative intensity when compared to the mass spectra corresponding to the overnight labeled samples. Actually, if we exclude these peptides, it is possible to confirm the trend observed for the standard protein samples: the higher the peptide mass, the lower the percentage of double ^{18}O-incorporation.

CONCLUSIONS

The results obtained show that the ultrasonic probe is capable of accelerating the labeling reaction from 12 h, the classic overnight methodology, to only 120 s without compromising the labeling efficiency. Yet, the labeling degree, i.e. the percentage of double ^{18}O-labeled peptides, was lower than that obtained with the classic methodology, especially for larger peptides. It was also found that the use of an ultrasonic probe is not recommended for the acceleration of the labeling reaction when the ultrasonication time is higher than 120 s, at least with the conditions reported here, because the aerosol formation, sample overheating and uncontrolled secondary reactions, that occur during ultrasonication at high intensities, compromise the double ^{18}O-incorporation at the carboxyl group of the peptide.

Regarding the sonoreactor, the results obtained from 30 to 120 s were similar to the ones obtained with the direct ultrasonication method, but in contrast to the ultrasonic probe, as the ultrasonication time is increased, higher labeling efficiencies and higher double labeling yields are obtained. Furthermore, the sonoreactor technology has some advantages over the ultrasonic probe: (i) it provides indirect and less intense ultrasonic energy, preventing aerosol formation; (ii) no sample overheating occurs, because the temperature of the water bath where ultrasonication takes place can be controlled; and last but not least (iii), the ultrasonication is performed in sealed vials, preventing sample contamination. The results achieved for the labeling degree ($^{18}O_2$ %) in just 15 min of ultrasonication were similar to the ones obtained previously with the overnight methodology. This was further confirmed with the labeling results obtained for ovalbumin.

When the ultrasonication was performed with different enzyme-to-protein (E:P) ratios the results showed that the labeling efficiency and the labeling degree were best with an E:P ratio of 1:40 (w/w). However, for low concentration protein samples, higher E:P ratios were required in order to achieve an acceptable double labeling yield, even when the classic methodology was performed.

Our results demonstrate that the isotopic labeling reaction can be performed in simple protein samples in only 15 min in a direct labeling approach using indirect ultrasonication provided by the sonoreactor. No intermediate drying steps are required in this workflow, which facilitate on-line approaches for protein quantitation. In addition, the sonoreactor has a higher sample throughput than the ultrasonic probe, which minimizes the sample treatment time and simplifies the overall workflow. However, when applied to a complex protein sample like human plasma, this technology was not capable of promoting efficient double ^{18}O-incorporation, thus compromising protein quantitation. Therefore, in the presence of this type of samples, two approaches can be used: (i) the decoupled labeling procedure in which peptides are double labeled in percentages higher than 95%;[36] or (ii) mathematical algorithms that measure the effective ^{18}O-incorporation rate due to variable enzyme substrate specificity during the labeling reaction and correct for the ^{18}O-abundance.[37,38]

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